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| <b>(21) International Application Number:</b> PCT/US94/14619<br><b>(22) International Filing Date:</b> 19 December 1994 (19.12.94)<br><b>(30) Priority Data:</b><br>08/168,809 17 December 1993 (17.12.93) US<br><b>(71) Applicant:</b> PIONEER HI-BRED INTERNATIONAL, INC.<br>[US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).<br><b>(72) Inventors:</b> PUTMAN, Rebecca, J.; Apartment 206, 5022 E.P. True Parkway, West Des Moines, IA 50265 (US). RAO, A., Gururaj; 4734 74th Street, Urbandale, IA 50322 (US).<br><b>(74) Agents:</b> YATES, Michael, E. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US). | <b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).<br><br><b>Published</b><br><i>With international search report.<br/>         Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |   |
| <b>(54) Title:</b> DERIVATIVES OF TACHYPLESIN HAVING INHIBITORY ACTIVITY TOWARDS PLANT PATHOGENIC FUNGI<br><br><b>(57) Abstract</b><br><p>Certain specific derivatives of the 17-amino acid peptide tachyplesin has been found to have antimicrobial activity against plant pathogens. In a preferred embodiment, plant resistance to diseases caused by plant pathogens which are susceptible to these peptides is produced by inserting into the cells of a plant a gene whose expression causes production of one or more of the peptides of this invention in the plant in antimicrobial amounts.</p>   |   |   |

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DERIVATIVES OF TACHYPLESIN HAVING INHIBITORY  
ACTIVITY TOWARDS PLANT PATHOGENIC FUNGI

Technical Field

5        This invention relates to compositions and methods for  
killing fungi which are harmful to plants, and materials and  
methods for imparting disease resistance to plants.

Background of the Invention

10       Numerous fungi and bacteria are serious pests of common  
agricultural crops. One method of controlling diseases has  
been to apply antimicrobial organic or semiorganic chemicals  
to crops or seeds. This method has numerous, art-recognized  
problems. A more recent method of controlling pathogenic  
15       organisms which are typically natural competitors or  
inhibitors of the troublesome microorganisms. However, it  
is difficult to apply biological control organisms to large  
areas, and even more difficult to assure that those living  
organisms will persist in the treated area for an extended  
20       period. Still more recently, techniques in recombinant DNA  
have provided the opportunity to insert into plant cells  
cloned genes which express peptides of interest, some of  
which are antimicrobial. This technology has given rise to  
additional concerns about eventual microbial resistance to  
25       well-known, naturally occurring antimicrobials, particularly  
in the face of heavy selection pressure, which may occur in  
some areas. Thus, a continuing need exists to identify  
naturally occurring antimicrobial compounds which can be  
formed by plant cells, either directly or indirectly, by  
30       translation of a single structural gene.

      European Patent Application 204,590, based upon U.S.  
Patent application Serial No. 725,368, describes a method of  
genetically modifying a plant cell to control expression of  
heterologous foreign structure genes. In the method, the  
35       plant cell is transformed to contain a pRi T-DNA promoter  
and a heterologous foreign structural gene, the promoter and

the structural gene being in such position and orientation with respect to one another that the structural gene is expressible in a plant cell under control of the promoter.

Likewise, European Patent Application 186,425, based upon U.S. patent application Serial No. 685,824, describes a recombinant DNA expression vector which comprises (a) a transcription unit, flanked by T-DNA border sequences, which comprises a promoter and associated amino terminal region encoding sequences and a terminator signal sequence in which the sequences are derived from one or more genes which are naturally expressed in a plant cell, and (b) an antibiotic resistance gene-encoding sequence located between the promoter and associated amino-terminal region-encoding sequence and the terminator sequence and (c) a DNA fragment containing a replicon that is functional in *Agrobacterium*.

PCT application 8807087, based upon U.S. patent application 168,109, discloses a recombinant virus expression system comprising a *Heliothis* polyhedrin promoter and a nucleotide sequence encoding a heterologous peptide or protein, which may have antimicrobial activity.

Akaji, K. et al., Chem. Pharm. Bull. (Tokyo) 37(10):2661-2664 (1989), reports the syntheses of three peptides from horseshoe crab, including tachyplesin I and II.

Kawano, K. et al., J. Biol. Chem. 265(26):15365-15367 (1990), reports the isolation of tachyplesin I from hemocytes of the horseshoe crab, and determination of its beta-sheet structure.

Miyata, T. et al., J. Biochem (Tokyo) 106(4):663-669 (1989), discloses isolation of tachyplesin II from horseshoe crab and elucidation of its structure and biological activity.

Muta, T. et al., J. Biochem (Tokyo) 108(2):261-266 (1990), discloses isolation of tachyplesin III and a processing intermediate of its precursor.

Nakamura, T. et al., J. Biol. Chem. 263(32):16709-16713 (1988), discusses isolation and chemical structure of tachyplesin.

Niwa, M. et al., First Scientific Meeting of the Japanese Association for Developmental and Comparative Immunology (Jadci), Tokyo, Japan, November 14 (2):2-3 (1990) deals with the antimicrobial activities of tachyplesin isopeptides.

Shieh, T.C. et al., FEBS (Fed. Eur. Biochem. Soc.) Lett. 252(1-2):121-124 (1989) discloses synthesis and properties of tachyplesin I.

Shigenaga, T. et al., J. Biol. Chem. 265(34):21350-21354 (1990) describes cloning of cDNA for the peptide precursor of tachyplesin and the cellular localization of the peptide in the horseshoe crab.

Japanese laid-open patent application 02207098, assigned to Taiyo Fishery KK, discloses a composition having high specificity to beta-glucan. The composition includes an amoebocyte lysate from *Lumulina* and blood cell membrane proteins including tachyplesins I and II.

Japanese laid-open patent application 02204500, also assigned to Taiyo Fishery KK, discloses an article insoluble carrier for removing pyrogens from fluids. The article is made by bonding crab peptides identified as tachyplesins I and II to a water-insoluble carrier.

Prior copending, commonly assigned application Serial No. 07/802,794, filed December 6, 1991, "Peptide with Inhibitory Activity Towards Plant Pathogenic Fungi", the European counterpart of which was published June 6, 1993, relates to the discovery that tachyplesins also have activity against fungal pathogens of common crop plants.

The disclosures of the foregoing references are hereby incorporated herein in their entirety by reference to show the state of the art.

Disclosure of the Invention

It has now been determined that certain specific derivatives of the peptide tachyplesin have potent antifungal activity against common plant pathogens.

5 Tachyplesin is a small peptide originally isolated from the hemocytes of the horseshoe crab, Tachyplesus tridentatus. The native peptide has 17 amino acids, six of which are cysteines. Tachyplesin's conformation consists of an antiparallel  $\beta$ -sheet connected by a  $\beta$ -turn. Five bulky

10 hydrophobic side groups are localized on one side of the plane and six cationic side groups are distributed at the tail part of the molecule.

The compounds of this invention have amino acid sequences indicated in the Sequence Listing as Sequence I.D. Nos. 1 through 3 and 5 through 14. In single-letter code preferred by biochemists, they are KWLFRVNGSSGKYRRQR (SEQUENCE I.D. NO. 1), KWLFRVNFRGIKYRRQR (SEQUENCE I.D. NO. 2), KWLFRVNQLMFKYRRQR (SEQUENCE I.D. NO. 3), KWAFRVAYRGIAYRRAR (SEQUENCE I.D. NO. 5) KWLFRVNYRGIKYRRQR

20 (SEQUENCE I.D. NO. 6), KWLFRVTYRGIKYRRQR (SEQUENCE I.D. NO. 7), KWLFRVNYRGIKYRRQRGIGAVLLKVLTTG (SEQUENCE I.D. NO. 8), KWLFRVNYRGIKYRRQRGSGGIGAVLKVLTTGL (SEQUENCE I.D. NO. 9), KYLFRVNYRGIKYRRQR (SEQUENCE I.D. NO. 10), KFLFRVNYRGIKYRRQR (SEQUENCE I.D. NO. 11), RYLFRVNYRGIKYRRQR (SEQUENCE I.D. NO. 12), KYQFRVNYRGIKYRRQR (SEQUENCE I.D. NO. 13), RYQFRVNYRGIKYRRQR (SEQUENCE I.D. NO. 14), KWLFRVNYRGIKYRRQRKDEL (SEQUENCE I.D. NO. 15), KWCFRVCYRGICYRRRCRGSTSGKWCFRVCYRGICYRRCR (SEQUENCE I.D. NO. 16), KWCFKVCYRGICYKKCK (SEQUENCE I.D. NO. 17),

30 KWKFRVKYRGIKYRRKR (SEQUENCE I.D. NO. 18), KWLFRVLYRGILYRRLR (SEQUENCE I.D. NO. 19), KWLFRVNKYRRQR (SEQUENCE I.D. NO. 20), and KWRFRVRYRGIEYRRER (SEQUENCE I.D. NO. 21), respectively. SEQUENCE I.D. NO. 4 is natural tachyplesin, with the sequence KWCFRVCYRGICYRRCR. All sequences are

35 indicated in the amino-to-carboxy direction.

These peptides are particularly active against the following group of plant pathogenic fungi and seed pathogens: *Fusarium graminearum*, *Fusarium moniliforme*, *Sclerotinia sclerotiorum*, *Sclerotinia trifoliorum*, and *Aspergillus flavus*. All of these fungi have significant economic impact. They produce crop losses through ear mold damage, and are also responsible for contamination of grain with aflatoxins and fumonisins.

Thus, this invention provides a method for killing and/or inhibiting susceptible plant pathogens, including microorganisms selected from the group listed above comprising the step of introducing into the environment of the organisms an antimicrobial amount of one or more peptides of this invention. "Killing" is meant here in the usual sense and "inhibiting" is used here in the sense commonly understood in antimicrobial susceptibility testing, namely, a reduction in growth and/or reproduction of the microorganism according to standard measurements, such as the Minimum Inhibitory Concentration, or MIC. Reference may be had to standard published texts, such as *Antimicrobial Susceptibility Testing*, 3rd Ed., written by the staff of the National Committee for Clinical Laboratory Standards (1991) and the multivolume set *Performance Standards for Antimicrobial Susceptibility Tests* published by the same group; and *Manual of Clinical Microbiology*, 5th ed., Balows, A. et al. Eds., pp. 1059-1202 (American Society for Microbiology, Washington, D.C., 1991) the disclosures of all of which are hereby incorporated by reference in their entirety.

These compounds can be effectively applied to plants infested with the microorganisms by spray, dust or other formulation common to the antimicrobial arts. Alternatively, the peptide can be incorporated into the tissues of a susceptible plant so that in the course of infesting the plant the pathogens will be exposed to antimicrobial amounts of the peptide. Known methods of

doing this are to incorporate the peptide in a non-phytotoxic vehicle which is adapted for systemic administration to the susceptible plants, such as an endphytic bacterium or a lipid vesicle or microsphere.

See, for example, U.S. 5,157,207, issued October 20, 1992 to Carlson, et al., the disclosures of which are hereby incorporated by reference, directed to modified plants containing bacterial inoculants. According to the '207 patent, certain types of microorganisms, such as hybrid agricultural-chemical-producing endosymbiotic microorganisms, can colonize the interior of plants and provide useful agricultural chemicals, such as pesticides, to the plants. Certain microbial endophytes are capable of inducing enhanced resistance in a host to phytopathogens. The bacteria that are suitable for use in such a method in this invention belong to a species of bacteria that is capable of replicating in the interior tissues of a plant and of producing the desired compound of this invention. Under normal field conditions, the bacterium does not ordinarily inhabit the seed or the plant into which the bacterium is introduced. Such a bacterium can be a gram-positive bacterium, a gram-negative bacterium or a species of actinomycetes. The bacterium is modified by genetic engineering techniques to incorporate the gene coding for the compound of this invention. In a preferred embodiment, the bacterial cell is a genetically modified species of either *Corynebacteria*, *Clavibacter*, *Pseudomonas*, *Xanthomonas* or *Erwinia*, the corynebacteria and clavibacters being as defined in Davis M. J. et al. (1984), loc. cit. In a particularly preferred embodiment, the bacterial cell is a strain of *Clavibacter xyli*. In a most preferred embodiment, the bacterial cell is a *Clavibacter xyli* subspecies *cynodontis*.

Reference also may be had to U.S. 5,252,348, issued  
35 October 12, 1993 and directed to "Artificial viral  
envelopes", the disclosures of which are also hereby



incorporated herein by reference. According to the '348 patent, lipid vesicles of the patent can be used to transfer biological material to plant cells.

Further reference may be had to U.S. 5,071,654, issued  
5 December 10, 1991, the disclosures of which are hereby  
incorporated by reference and which are directed to  
insecticidal compositions involving a phospholipid vesicle  
with insect midgut brush border and a *Bacillus thuringiensis*  
protein endotoxin incorporated therein and an  
10 agriculturally-acceptable carrier. Such phospholipid  
vesicle compositions are stated to provide a vehicle for the  
application of delta endotoxin to plants. The vesicles are  
stated to act in much the same fashion as liposomes do in  
vertebrates, to facilitate the delivery of the toxins to the  
15 target midgut cells in susceptible insects.

Reference may also be had to U.S. 5,034,322, issued  
July 23, 1991 to Rogers, et. al., the disclosures of which  
are hereby incorporated by reference and which are directed  
to chimeric genes suitable for expression in plant cells,  
20 but which discusses methods capable of inserting the  
chimeric genes of this invention into plant cells, although  
the reported transformation efficiencies achieved to date by  
such methods have been low. Discussed methods include use  
of lipid vesicles, also called liposomes, from which DNA  
25 may be taken up by plant cells.

Still further reference may be had to U.S. 4,588,578,  
issued May 13, 1986 to Fountain, et. al. and directed to  
monophasic vesicles for delivery of antimicrobials and other  
compounds to plants, and U.S. 4,522,803, issued June 11,  
30 1985 to Lenk, et. al. and directed, *inter alia*, to methods  
for treatment of infections in plants, comprising  
administering stable plurilamellar vesicles containing a  
compound effective for treating said infection. The  
disclosures of these patents are also hereby incorporated by  
35 reference.

The foregoing and similar methods are commonly employed with insecticidal materials which are designed to attack chewing insects and are well within the purview of one of ordinary skill in the art of insecticide and larvicide formulation and are fully contemplated in the practice of this invention. However, genes which code for the peptides of this invention can be readily synthesized using automated methods, cloned, inserted into an appropriate expression cassette, and introduced directly into cells of a susceptible plant species. Accordingly, an especially preferred embodiment of this method involves inserting into the genome of the plant a DNA sequence coding for one or more of the peptides of this invention in proper reading frame, together with transcription initiator and promoter sequences active in the plant. Transcription and translation of the DNA sequence(s) under control of the regulatory sequences causes expression of the peptide sequence(s) at levels which provide an antimicrobial amount of the peptide(s) in the tissues of the plant which are normally infected by the pathogens.

With a working knowledge of conventional techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques known and commonly employed by those skilled in the art (See, for example, R. Wu, ed. (1979) Meth. Enzymol. 68; R. Wu et al., eds. (1983) Meth. Enzymol. 100, 101; L. Grossman and K. Moldave, eds. (1980) Meth. Enzymol. 65; J. H. Miller (1972) Experiments in Molecular Genetics; R. Davis et al. (1980) Advanced Bacterial Genetics; R. F. Schleif and P. C. Wensink (1982) Practical Methods in Molecular Biology; and T. Manniatis et al. (1982) Molecular Cloning.), one of ordinary skill can employ any suitable gene construct containing the structural genes coding for the peptides of this invention.

The plant is preferably a plant susceptible to infection and damage by one or more of *Fusarium graminearum*, *Fusarium moniliforme*, *Sclerotinia sclerotiorum*, *Sclerotinia trifoliorum*, or *Aspergillus flavus*. These include corn (*Zea mays*) and sorghum (*Sorghum bicolor*). However, this is not to be construed as limiting, inasmuch as these species are among the most difficult commercial crops to reliably transform and regenerate, and these microorganisms also infect many other crops. Thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, if they are found to be susceptible to the plant pests listed hereinabove, including, without limitation, species from the genera *Allium*, *Antirrhinum*, *Arabidopsis*, *Arachis*, *Asparagus*, *Atropa*, *Avena*, *Beta*, *Brassica*, *Browallia*, *Capsicum*, *Cicer*, *Cicla*, *Citrullus*, *Citrus*, *Cucumis*, *Cucurbita*, *Datura*, *Daucus*, *Digitalis*, *Fagopyrum*, *Fragaria*, *Geranium*, *Glycine*, *Gossypium*, *Helianthus*, *Hordeum*, *Hemerocallis*, *Lactuca*, *Lens*, *Lolium*, *Lotus*, *Lycopersicon*, *Majorana*, *Manihot*, *Medicago*, *Nasturtium*, *Nicotiana*, *Oryza*, *Pelargonium*, *Persea*, *Petunia*, *Phaseolus*, *Pisum*, *Ranunculus*, *Raphanus*, *Ricinus*, *Saccharum*, *Secale*, *Senecio*, *Setaria*, *Solanum*, *Spinacia*, *Trifolium*, *Triticum*, *Bromus*, *Cichorium*, *Hyoscyamus*, *Linum*, *Nemesia*, *Panicum*, *Onobrychis*, *Pennisetum*, *Salpiglossis*, *Sinapis*, *Trigonella*, and *Vigna*. Preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean.

The DNA sequence which when expressed imparts antimicrobial activity is a structural gene which codes for at least one of the peptides of this invention.

Given the amino acid sequences provided herein, any of several translationally equivalent synthetic DNA sequences can then be prepared which code for the sequences of amino acids using commercially available software, such as PC

Gene<sup>TM</sup> or GCG<sup>TM</sup>, and this synthetic sequence can be inserted into an appropriate plant expression cassette.

Likewise, numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the cDNA of the present invention can be inserted is the pPHI414 plasmid developed by Beach et al. of Pioneer Hi-Bred International, Inc., Johnston, IA, as described in U.S. Patent Application Serial No. 387,739, filed August 1, 1989, and its continuation-in-part, Serial No. 785,648, filed October 31, 1991, the disclosures of which are hereby incorporated herein by reference in their entirety. Highly preferred plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. Such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance,

which then serve as markers to identify and separate transformed cells. Preferred markers/selection agents include kanamycin, chlorosulfuron, phosphonothricin, hygromycin and methotrexate. A cell in which the foreign genetic material  
5 in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant."

A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule which is not a part of the chromosomes of the cell.

10 According to this invention, the genetic construct will contain (a) a first genetic sequence coding for at least one peptide of this invention, and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. Typically, the regulatory sequences will  
15 be selected from the group comprising promoters and terminators. The regulatory sequences may be from autologous or heterologous sources.

Promoters that may be used in the genetic sequence include nos, ocs, napin, phaseolin and CaMV promoters, as  
20 well as others specifically discussed herein.

An efficient plant promoter that may be used is an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-biphosphate  
25 carboxylase from soybean (Berry-Lowe et al, J. Molecular and App. Gen., 1:483-498 (1982)), and the promoter of the cholorophyll a-b binding protein. These two promoters are known to be light-induced in eukaryotic plant cells (see, for example, Genetic Engineering of Plants, An Agricultural  
30 Perspective, A. Cashmore, Pelham, New York, 1983, pp. 29-38, G. Coruzzi et al., J. Biol. Chem., 258:1399 (1983), and P. Dunsmuir, et al., J. Molecular and App. Gen., 2:285 (1983)).

The expression cassette comprising the structural gene for one or more peptides of this invention operably linked  
35 to the desired control sequences can be ligated into a suitable cloning vector. In general, plasmid or viral

(bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. The cloning vector will typically carry a replication origin, as well as specific genes that are  
5 capable of providing phenotypic selection markers in transformed host cells. Typically, genes conferring resistance to antibiotics or selected herbicides are used. After the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of  
10 cells can be isolated by selection on the basis of these markers.

Typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the  
15 vector containing the antimicrobial peptide gene can be isolated in significant quantities for introduction into the desired plant cells. Host cells that can be used in the practice of this invention include prokaryotes, including bacterial hosts such as *E. coli*, *S. typhimurium*, and  
20 *Serratia marcescens*. Eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. Since these hosts are also microorganisms, it will be essential to ensure that plant promoters which do not cause expression of the peptide in bacteria are used in the vector, or that a  
25 host microorganism which is resistant to is used. However, a particular benefit of the peptides of this invention is their relatively low toxicity to common bacterial hosts such as *E. coli* and *Agrobacterium tumefaciens*. By comparison, tachyplesin shows significant toxicity to *E. coli* even at  
30 the lowest effective antifungal concentrations.

The isolated cloning vector will then be introduced into the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, bombardment, and microinjection, into cells from monocotyledonous or dicotyledonous plants, in cell or tissue culture,  
35 to provide transformed plant cells containing as foreign DNA

at least one copy and preferably more than one copy of the DNA sequence of the plant expression cassette. Preferably, the monocotyledonous species will be selected from maize, sorghum, wheat and rice, and the dicotyledonous species will  
5 be selected from soybean, alfalfa, tobacco and tomato. Using known techniques, protoplasts can be regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for one or more of the peptides of this invention. Accordingly, a  
10 highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette of this invention as described above.

Finally, this invention provides methods of imparting  
15 resistance to diseases caused by microorganisms selected from *Fusarium graminearum*, *Fusarium moniliforme*, *Sclerotinia sclerotiorum*, *Sclerotinia trifoliorum*, and *Aspergillus flavus* to plants of a susceptible taxon, comprising the steps of:

- 20 a) culturing regenerable cells or tissues from at least one plant from the taxon,
- b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a gene which codes for at least one peptide of this  
25 invention, operably linked to plant regulatory sequences which cause the expression of peptide(s) in the cells, and
- c) regenerating disease-resistant whole plants from the cell or tissue culture. Once whole plants have been obtained, they can be sexually or clonally reproduced in  
30 such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

Alternatively, once a single transformed plant has been obtained by the foregoing recombinant DNA method, conventional plant breeding methods can be used to transfer the  
35 structural gene for the antimicrobial peptide and associated

regulatory sequences via crossing and backcrossing. Such intermediate methods will comprise the further steps of

a) sexually crossing the disease-resistant plant with a plant from the disease-susceptible taxon;

5 b) recovering reproductive material from the progeny of the cross; and

c) growing disease-resistant plants from the reproductive material. Where desirable or necessary, the agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to include the further steps of repetitively:

a) backcrossing the disease-resistant progeny with disease-susceptible plants from the susceptible taxon; and

15 b) selecting for expression of antimicrobial activity (or an associated marker gene) among the progeny of the backcross, until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with the gene imparting antimicrobial activity.

By the term "taxon" herein is meant a unit of botanical classification of genus or lower. It thus includes genus, species, cultivars, varieties, variants, and other minor taxonomic groups which lack a consistent nomenclature.

It will also be appreciated by those of ordinary skill that the plant vectors provided herein can be incorporated into *Agrobacterium tumefaciens*, which can then be used to transfer the vector into susceptible plant cells, especially those from dicotyledonous species. Thus, this invention provides a method for imparting antimicrobial activity and disease resistance in *Agrobacterium tumefaciens*-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with *Agrobacterium tumefaciens*, a plasmid of which has been modified to include a plant expression cassette of this invention.

#### Human and Veterinary Pharmaceutical Use

35 This invention also provides methods of treating and preventing infection by susceptible organisms in a human or



lower animal host in need of such treatment, which method comprises administration to the human or lower animal host in need of such treatment a therapeutically effective amount of a polypeptide of this invention or a composition  
5 containing one or more of the polypeptides. The polypeptides of the present invention may be administered parenterally, by inhalation spray, rectally or topically in dosage unit formulations containing conventional nontoxic, pharmaceutically acceptable carriers, adjuvants and vehicles  
10 as desired. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intraarticular and intrathecal injection and infusion techniques. As with other polypeptides, the polypeptides of this invention are not known to be active orally.

15 Total daily dose of the compounds of this invention administered to a host in single or divided doses may be in amounts, for example, of from 1 to 2000 mg/kg body weight daily and more usually 50 to 500 mg/kg. Dosage unit compositions may contain such amounts or fractions or  
20 submultiples thereof as appropriate to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health,  
25 sex, diet, time of administration, route of administration, rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

This invention also provides pharmaceutical compositions in unit dosage form, comprising an effective amount of a  
30 compound of this invention in combination with a conventional pharmaceutical carrier. As used herein, the term "pharmaceutical carrier" means a solid or liquid filler, diluent or encapsulating material. Some examples of the materials which can serve as pharmaceutical carriers are  
35 sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its

derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil, 5 safflower oil, sesame oil, olive oil, corn oil and soybean oil; polyols such as propylene glycol, glycerin, sorbitol, mannitol and polyethylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; 10 pyrogen-free water; isotonic saline; Ringer's solution, ethyl alcohol and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants such as sodium lauryl sulfate and magnesium stearate, as 15 well as coloring agents, release agents, coating agents, and perfuming agents and preservatives can also be present in the compositions, according to the desires of the formulator. The amount of active ingredient that may be combined with the carrier materials to produce a single 20 dosage form will vary depending upon the host treated and the particular mode of administration.

By "therapeutically effective amount" herein is meant an amount of either polypeptide or combination thereof sufficient to provide antifungal activity so as to alleviate 25 or prevent infection by susceptible organisms in the human or lower animal being treated at a reasonable benefit/risk ratio attendant with any medical treatment.

The following description further exemplifies the compositions of this invention and the methods of making and 30 using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

Industrial ApplicabilityExample 1Antifungal Testing

Peptide samples for this and the other Examples were  
5 synthesized from the predetermined amino acid sequence and  
structure, according to methods reported in the literature,  
specifically the "Fastmoc" method described by C.G. Fields,  
et al., Peptide Res., Vol. 4 pp.95-101 (1991). Since the  
peptides of this invention are new but the methods of their  
10 synthesis are routine, the methods of synthesis per se are  
not considered a part of this invention.

Minimum Inhibitory Concentrations (MIC) and Minimum  
Complete Inhibitory Concentrations (MCIC) in µg/mL were  
determined for each of the compounds of this invention using  
15 the published methods of Duvick, J.P. et al, J. Biol. Chem.,  
267, 18814-18820 (1992). Results were as follows:

SEQUENCE I.D. NO. 1

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
|    | <u>Aspergillus flavus</u>   | 60         | >80         |
| 20 | <u>Fusarium graminearum</u> | 20         | 60          |
|    | <u>Fusarium moniliforme</u> | 10         | 40          |

SEQUENCE I.D. NO. 2

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 25 | <u>Aspergillus flavus</u>   | 20         | >80         |
|    | <u>Fusarium graminearum</u> | 20         | 40          |
|    | <u>Fusarium moniliforme</u> | 10         | 20          |

SEQUENCE I.D. NO. 3

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 30 | <u>Aspergillus flavus</u>   | 5          | 60          |
|    | <u>Fusarium graminearum</u> | 10         | 10          |
|    | <u>Fusarium moniliforme</u> | 2.5        | 10          |

## SEQUENCE I.D. NO. 5

|   | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|---|-----------------------------|------------|-------------|
|   | <u>Aspergillus flavus</u>   | NA         | NA          |
|   | <u>Fusarium graminearum</u> | NA         | NA          |
| 5 | <u>Fusarium moniliforme</u> | NA         | NA          |

## SEQUENCE I.D. NO. 6

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
|    | <u>Aspergillus flavus</u>   | 38         | 61          |
| 10 | <u>Fusarium graminearum</u> | 25         | 48          |
|    | <u>Fusarium moniliforme</u> | 16         | 48          |

## SEQUENCE I.D. NO. 7

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 15 | <u>Aspergillus flavus</u>   | 40         | >80         |
|    | <u>Fusarium graminearum</u> | 20         | 30          |
|    | <u>Fusarium moniliforme</u> | 20         | 40          |

## SEQUENCE I.D. NO. 8

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 20 | <u>Aspergillus flavus</u>   | 38         | >80         |
|    | <u>Fusarium graminearum</u> | 13         | 18          |
|    | <u>Fusarium moniliforme</u> | 13         | 35          |

## 25 SEQUENCE I.D. NO. 9

|  | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|--|-----------------------------|------------|-------------|
|  | <u>Aspergillus flavus</u>   | NA         | NA          |
|  | <u>Fusarium graminearum</u> | 40         | 80          |
|  | <u>Fusarium moniliforme</u> | 40         | >80         |

30

## SEQUENCE I.D. NO. 10

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
|    | <u>Aspergillus flavus</u>   | 50         | >80         |
|    | <u>Fusarium graminearum</u> | 22         | 58          |
| 35 | <u>Fusarium moniliforme</u> | 20         | 63          |

## SEQUENCE I.D. NO. 11

|   | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|---|-----------------------------|------------|-------------|
|   | <u>Aspergillus flavus</u>   | 40         | >80         |
|   | <u>Fusarium graminearum</u> | 40         | 80          |
| 5 | <u>Fusarium moniliforme</u> | 20         | 80          |

## SEQUENCE I.D. NO. 12

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
|    | <u>Aspergillus flavus</u>   | NA         | NA          |
| 10 | <u>Fusarium graminearum</u> | 80         | >80         |
|    | <u>Fusarium moniliforme</u> | 80         | >80         |

## SEQUENCE I.D. NO. 13

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 15 | <u>Aspergillus flavus</u>   | 28         | 200         |
|    | <u>Fusarium graminearum</u> | 32         | 58          |
|    | <u>Fusarium moniliforme</u> | 13         | 68          |

## SEQUENCE I.D. NO. 14

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 20 | <u>Aspergillus flavus</u>   | 35         | 200         |
|    | <u>Fusarium graminearum</u> | 32         | 68          |
|    | <u>Fusarium moniliforme</u> | 18         | 68          |

## 25 SEQUENCE I.D. NO. 15

|  | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|--|-----------------------------|------------|-------------|
|  | <u>Aspergillus flavus</u>   | 80         | >80         |
|  | <u>Fusarium graminearum</u> | 30         | 80          |
|  | <u>Fusarium moniliforme</u> | 30         | >80         |

30

## SEQUENCE I.D. NO. 16

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
|    | <u>Aspergillus flavus</u>   | 32         | >64         |
|    | <u>Fusarium graminearum</u> | 12         | 32          |
| 35 | <u>Fusarium moniliforme</u> | 16         | 64          |

## SEQUENCE I.D. NO. 17

|   | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|---|-----------------------------|------------|-------------|
|   | <u>Aspergillus flavus</u>   | 25         | 93          |
|   | <u>Fusarium graminearum</u> | 7.5        | 25          |
| 5 | <u>Fusarium moniliforme</u> | 12         | 48          |

## SEQUENCE I.D. NO. 18

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
|    | <u>Aspergillus flavus</u>   | 20         | >80         |
| 10 | <u>Fusarium graminearum</u> | 10         | 20          |
|    | <u>Fusarium moniliforme</u> | 5          | 20          |

## SEQUENCE I.D. NO. 19

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 15 | <u>Aspergillus flavus</u>   | 60         | >80         |
|    | <u>Fusarium graminearum</u> | 10         | 80          |
|    | <u>Fusarium moniliforme</u> | 3.75       | 80          |

## SEQUENCE I.D. NO. 20

|    | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|----|-----------------------------|------------|-------------|
| 20 | <u>Aspergillus flavus</u>   | 20         | 80          |
|    | <u>Fusarium graminearum</u> | 20         | 40          |
|    | <u>Fusarium moniliforme</u> | 10         | 40          |

## 25 SEQUENCE I.D. NO. 21

|  | <u>Fungus</u>               | <u>MIC</u> | <u>MCIC</u> |
|--|-----------------------------|------------|-------------|
|  | <u>Aspergillus flavus</u>   | 20         | 80          |
|  | <u>Fusarium graminearum</u> | 20         | 20          |
|  | <u>Fusarium moniliforme</u> | 10         | 40          |

30 In these tables, NA = not active.

For comparison, tachyplesin and other known antimicrobial compounds have MICs as follows:

|                      |            | MIC $\mu\text{g/ml}$ ( $\mu\text{M}$ ) |            |
|----------------------|------------|--|------------|
| <u>Protein</u>       | <u>AFL</u> | <u>FGR</u>                             | <u>FMO</u> |
| tachyplesin<br>(2.8) | 6.25 (2.8) | 6.25 (2.8)                             | 6.25       |
| 5 defensin<br>(3)    | 11.7 (3)   | 2.6 (0.7)                              | 11.7       |
| hordothionin         |            | 15 (3) 5 (1)                           | 45 (9)     |
| CMIII<br>(4.6)       | 60 (15)    | 6.2 (1.6)                              | 18.2       |

10 Example 2Antibacterial Testing

Minimum Inhibitory Concentrations (MIC) and Minimum Complete Inhibitory Concentrations (MCIC) in  $\mu\text{g/mL}$  against selected species of bacteria were determined for each of the compounds of this invention. Results were as follows:

SEQUENCE I.D. NO. 1

| <u>Bacterium</u>               | <u>MIC</u> | <u>MCIC</u> |
|--------------------------------|------------|-------------|
| <u>E. coli</u>                 | 6.25       | >100        |
| 20 <u>E. stewartii</u><br>>100 |            | 6.25        |
| <u>LBA 4404</u>                | 50         | >100        |
| <u>C. nebraskense</u><br>25    |            | 3.13        |
| 25 <u>B. pumillus</u>          | >100       |             |

SEQUENCE I.D. NO. 2

| <u>Bacterium</u>              | <u>MIC</u> | <u>MCIC</u> |
|-------------------------------|------------|-------------|
| <u>E. coli</u>                | 6.25       | >100        |
| <u>E. stewartii</u><br>>100   |            | 6.25        |
| 30 <u>LBA 4404</u>            | 6.25       | 25          |
| <u>C. nebraskense</u><br>12.5 |            | 6.25        |
| <u>B. pumillus</u>            | 25         | >100        |

|                     |                       |            |             |
|---------------------|-----------------------|------------|-------------|
| SEQUENCE I.D. NO. 3 |                       |            |             |
|                     | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                     | <u>E. coli</u>        | 3.13       | >100        |
|                     | <u>E. stewartii</u>   |            | 3.13        |
| 5                   | >100                  |            |             |
|                     | <u>LBA 4404</u>       | 50         | 100         |
|                     | <u>C. nebraskense</u> |            | nt          |
|                     | <u>B. pumillus</u>    | 12.5       | >100        |
| SEQUENCE I.D. NO. 5 |                       |            |             |
| 10                  | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                     | <u>E. coli</u>        | nt         |             |
|                     | <u>E. stewartii</u>   |            | nt          |
|                     | <u>LBA 4404</u>       | nt         |             |
|                     | <u>C. nebraskense</u> |            | nt          |
| 15                  | <u>B. pumillus</u>    | nt         |             |
| SEQUENCE I.D. NO. 6 |                       |            |             |
|                     | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                     | <u>E. coli</u>        | 100        | >100        |
|                     | <u>E. stewartii</u>   |            | 6.25        |
| 20                  | >100                  |            |             |
|                     | <u>LBA 4404</u>       | 6.25       | 50          |
|                     | <u>C. nebraskense</u> |            | 6.25        |
|                     | 12.5                  |            |             |
|                     | <u>B. pumillus</u>    | 50         | >100        |
| 25                  | SEQUENCE I.D. NO. 7   |            |             |
|                     | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                     | <u>E. coli</u>        | 100        | >100        |
|                     | <u>E. stewartii</u>   |            | 6.25        |
|                     | >100                  |            |             |
| 30                  | <u>LBA 4404</u>       | 50         | 100         |
|                     | <u>C. nebraskense</u> |            | 6.25        |
|                     | 25                    |            |             |
|                     | <u>B. pumillus</u>    | 25         | >100        |



|                     |                       |            |             |
|---------------------|-----------------------|------------|-------------|
| SEQUENCE I.D. NO. 8 |                       |            |             |
|                     | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                     | <u>E. coli</u>        | 25         | >100        |
|                     | <u>E. stewartii</u>   |            | 6.25        |
| 5                   | >100                  |            |             |
|                     | <u>LBA 4404</u>       | 6.25       | >100        |
|                     | <u>C. nebraskense</u> |            | 6.25        |
|                     | 12.5                  |            |             |
|                     | <u>B. pumillus</u>    | 50         | >100        |
| 10                  | SEQUENCE I.D. NO. 9   |            |             |
|                     | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                     | <u>E. coli</u>        | 12.5       | >100        |
|                     | <u>E. stewartii</u>   |            | 12.5        |
|                     | >100                  |            |             |
| 15                  | <u>LBA 4404</u>       | 100        | >100        |
|                     | <u>C. nebraskense</u> |            | 12.5        |
|                     | 12.5                  |            |             |
|                     | <u>B. pumillus</u>    | 50         | >100        |
|                     | SEQUENCE I.D. NO. 10  |            |             |
| 20                  | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                     | <u>E. coli</u>        | 100        | >100        |
|                     | <u>E. stewartii</u>   |            | 6.25        |
|                     | >100                  |            |             |
|                     | <u>LBA 4404</u>       | 25         | 100         |
| 25                  | <u>C. nebraskense</u> |            | 12.5        |
|                     | 12.5                  |            |             |
|                     | <u>B. pumillus</u>    | 50         | >100        |
|                     | SEQUENCE I.D. NO. 11  |            |             |
|                     | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
| 30                  | <u>E. coli</u>        | 50         | >100        |
|                     | <u>E. stewartii</u>   |            | 6.25        |
|                     | >100                  |            |             |
|                     | <u>LBA 4404</u>       | 12.5       | >100        |
|                     | <u>C. nebraskense</u> |            | 12.5        |
|                     | 12.5                  |            |             |
| 35                  | <u>B. pumillus</u>    | 100        | >100        |

|                      |                       |            |             |
|----------------------|-----------------------|------------|-------------|
| SEQUENCE I.D. NO. 12 |                       |            |             |
|                      | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                      | <u>E. coli</u>        | 100        | >100        |
|                      | <u>E. stewartii</u>   |            | 12.5        |
| 5                    | >100                  |            |             |
|                      | <u>LBA 4404</u>       | nt         | nt          |
|                      | <u>C. nebraskense</u> |            | nt nt       |
|                      | <u>B. pumillus</u>    | nt         | nt          |
| SEQUENCE I.D. NO. 13 |                       |            |             |
| 10                   | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                      | <u>E. coli</u>        | 50         | >100        |
|                      | <u>E. stewartii</u>   |            | 3.13        |
|                      | >100                  |            |             |
|                      | <u>LBA 4404</u>       | 50         | >100        |
| 15                   | <u>C. nebraskense</u> |            | 6.25        |
|                      | >100                  |            |             |
|                      | <u>B. pumillus</u>    | 6.25       | 100         |
| SEQUENCE I.D. NO. 14 |                       |            |             |
|                      | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
| 20                   | <u>E. coli</u>        | 100        | >100        |
|                      | <u>E. stewartii</u>   |            | 6.25        |
|                      | >100                  |            |             |
|                      | <u>LBA 4404</u>       | 3.13       | >100        |
|                      | <u>C. nebraskense</u> |            | 6.25        |
| 25                   | 100                   |            |             |
|                      | <u>B. pumillus</u>    | 6.25       | >100        |
| SEQUENCE I.D. NO. 15 |                       |            |             |
|                      | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                      | <u>E. coli</u>        | 50         | >100        |
| 30                   | <u>E. stewartii</u>   |            | 6.25        |
|                      | 50                    |            |             |
|                      | <u>LBA 4404</u>       | 6.25       | >100        |
|                      | <u>C. nebraskense</u> |            | 50 >100     |
|                      | <u>B. pumillus</u>    | 50         | >100        |

|                      |                       |            |             |
|----------------------|-----------------------|------------|-------------|
| SEQUENCE I.D. NO. 17 |                       |            |             |
|                      | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                      | <u>E. coli</u>        | 3.13       | >100        |
|                      | <u>E. stewartii</u>   |            | 3.13        |
| 5                    | 25                    |            |             |
|                      | <u>LBA 4404</u>       | 3.13       | 12.5        |
|                      | <u>C. nebraskense</u> |            | 3.13        |
|                      | 25                    |            |             |
|                      | <u>B. pumillus</u>    | 6.25       | 100         |
| 10                   | SEQUENCE I.D. NO. 18  |            |             |
|                      | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                      | <u>E. coli</u>        | 6.25       | 25          |
|                      | <u>E. stewartii</u>   |            | 6.25        |
|                      | 100                   |            |             |
| 15                   | <u>LBA 4404</u>       | 50         | >100        |
|                      | <u>C. nebraskense</u> |            | 6.25        |
|                      | 6.25                  |            |             |
|                      | <u>B. pumillus</u>    | >100       |             |
|                      | SEQUENCE I.D. NO. 19  |            |             |
| 20                   | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
|                      | <u>E. coli</u>        | 3.13       | 50          |
|                      | <u>E. stewartii</u>   |            | 12.5        |
|                      | 50                    |            |             |
|                      | <u>LBA 4404</u>       | 25         | 100         |
| 25                   | <u>C. nebraskense</u> |            | 3.13        |
|                      | 12.5                  |            |             |
|                      | <u>B. pumillus</u>    | 100        | >100        |
|                      | SEQUENCE I.D. NO. 20  |            |             |
|                      | <u>Bacterium</u>      | <u>MIC</u> | <u>MCIC</u> |
| 30                   | <u>E. coli</u>        | 3.13       | 3.13        |
|                      | <u>E. stewartii</u>   |            | 12.5        |
|                      | 50                    |            |             |
|                      | <u>LBA 4404</u>       | 50         | 50          |
|                      | <u>C. nebraskense</u> |            | 6.25        |
| 35                   | 25                    |            |             |
|                      | <u>B. pumillus</u>    | 100        | >100        |

| SEQUENCE I.D. NO. 21        |            |             |
|-----------------------------|------------|-------------|
| <u>Bacterium</u>            |            |             |
|                             | <u>MIC</u> | <u>MCIC</u> |
| <u>E. coli</u>              | 6.25       | 25          |
| <u>E. stewartii</u>         |            | 6.25        |
| 5                      12.5 |            |             |
| <u>LBA 4404</u>             | 25         | 50          |
| <u>C. nebraskense</u>       |            | 3.13        |
| 25                          |            |             |
| <u>B. pumillus</u>          | >100       |             |

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Pioneer Hi-Bred International, Inc.

5 (ii) TITLE OF INVENTION: DERIVATIVES OF TACHYPLESIN HAVING  
INHIBITORY ACTIVITY TOWARDS PLANT PATHOGENIC FUNGI

(iii) NUMBER OF SEQUENCES: 21

## (iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Des Moines  
(D) STATE: IA  
(E) COUNTRY: USA  
15 (F) ZIP: 50309

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
20 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## 25 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Yates, Michael E.  
(B) REGISTRATION NUMBER:  
(C) REFERENCE/DOCKET NUMBER: 0244 US

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 17 amino acids  
(B) TYPE: amino acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(iii) HYPOTHETICAL: NO  
5 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
Lys Trp Leu Phe Arg Val Asn Gly Ser Ser Gly Lys Tyr Arg Arg Gln  
1 5 10 15  
10 Arg

(2) INFORMATION FOR SEQ ID NO:2:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 17 amino acids  
15 (B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(iii) HYPOTHETICAL: NO  
20 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:  
Lys Trp Leu Phe Arg Val Asn Phe Arg Gly Ile Lys Tyr Arg Arg Gln  
1 5 10 15  
25 Arg

(2) INFORMATION FOR SEQ ID NO:3:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 17 amino acids  
30 (B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(iii) HYPOTHETICAL: NO  
35 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Trp Leu Phe Arg Val Asn Gln Leu Met Phe Lys Tyr Arg Arg Gln

1 5 10 15

Arg

5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Trp Cys Phe Arg Val Cys Tyr Arg Gly Ile Cys Tyr Arg Arg Cys

1 5 10 15

Arg

20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Trp Ala Phe Arg Val Ala Tyr Arg Gly Ile Ala Tyr Arg Arg Ala

1 5 10 15

Arg

35

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

5 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Trp Leu Phe Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln

1

5

10

15

Arg

15

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Trp Leu Phe Arg Val Thr Tyr Arg Gly Ile Lys Tyr Arg Arg Gln

1

5

10

15

Arg

30

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 amino acids

(B) TYPE: amino acid

35 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear



- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Trp Leu Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln Arg  
 1 5 10 15  
 Gly Ile Gly Ala Val Leu Leu Lys Val Leu Thr Thr Gly  
 20 25

10 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Trp Leu Phe Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln  
 1 5 10 15  
 Arg Gly Ser Thr Ser Gly Gly Ile Gly Ala Val Leu Lys Val Leu Thr  
 20 25 30

25 Thr Gly Leu  
 35

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

35

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Tyr Leu Phe Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln

1 5 10 15

Arg

5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Phe Leu Phe Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln

1 5 10 15

Arg

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg Tyr Leu Phe Arg Val Asn Tyr Arg Gly Ile Arg Tyr Arg Arg Gln

1 5 10 15

Arg

35

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

5 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Tyr Gln Phe Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln

1 5 10 15

Arg

15

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Tyr Gln Phe Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln

1 5 10 15

Arg

30

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids

(B) TYPE: amino acid

35 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO
- 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:  
Lys Trp Leu Phe Arg Val Asn Tyr Arg Gly Ile Lys Tyr Arg Arg Gln  
1 5 10 15  
Arg Lys Asp Glu Leu  
20
- 10 (2) INFORMATION FOR SEQ ID NO:16:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 39 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:  
Lys Trp Cys Phe Arg Val Cys Tyr Arg Gly Ile Cys Tyr Arg Arg Cys  
1 5 10 15  
Arg Gly Ser Thr Ser Gly Lys Trp Cys Phe Arg Val Cys Tyr Arg Gly  
20 25 30  
25 Ile Cys Tyr Arg Arg Cys Arg  
35
- (2) INFORMATION FOR SEQ ID NO:17:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 17 amino acids  
30 (B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: protein  
(iii) HYPOTHETICAL: NO  
35 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Lys Trp Cys Phe Lys Val Cys Tyr Arg Gly Ile Cys Tyr Lys Lys Cys

1 5 10 15

Lys

5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Lys Trp Lys Phe Arg Val Lys Tyr Arg Gly Ile Lys Tyr Arg Arg Lys

1 5 10 15

Arg

20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Lys Trp Leu Phe Arg Val Leu Tyr Arg Gly Ile Leu Tyr Arg Arg Leu

1 5 10 15

Arg

35

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

5 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Lys Trp Leu Phe Arg Val Asn Lys Tyr Arg Arg Gln Arg

1

5

10

## (2) INFORMATION FOR SEQ ID NO:21:

## 15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

25 Lys Trp Arg Phe Arg Val Arg Tyr Arg Gly Ile Glu Tyr Arg Arg Glu

1

5

10

15

Arg

## WHAT IS CLAIMED IS:

1. A peptide having the amino acid sequence of one of Sequence I.D. Nos. 1 through 3 or 6 through 21.
2. A nucleotide fragment which codes for a peptide  
5 having the amino acid sequence of one of Sequence I.D. Nos. 1 through 3 or 6 through 21.
3. A method for killing and inhibiting fungi comprising introducing into the environment of the pathogenic microorganisms an antimicrobial amount of at  
10 least one of the peptides having the amino acid sequence of one of Sequence I.D. Nos. 1 through 3 or 6 through 21.
4. A method according to Claim 3 for killing and inhibiting plant pathogens selected from *Fusarium graminearum*, *Fusarium moniliforme*, *Sclerotinia sclerotiorum*,  
15 *Sclerotinia trifoliorum*, and *Aspergillus flavus*.
5. A method according to Claim 4 wherein the environment of the pathogen is the tissues of a living plant.
6. A method according Claim 4 for protecting a plant against infection by pathogens selected from *Fusarium*  
20 *graminearum*, *Fusarium moniliforme*, *Sclerotinia sclerotiorum*, *Sclerotinia trifoliorum*, and *Aspergillus flavus* comprising inserting into the genome of the plant a sequence which codes for at least one peptide having the amino acid sequence of one of SEQUENCE I.D. NOS. 1 through 3 or 6  
25 through 21, in proper reading frame relative to transcription initiator and promoter sequences active in the plant to cause expression of the coding sequence at levels which provide a antimicrobial amount of the peptide or peptides in the tissues of the plant which are normally  
30 infected by the pathogens.
7. A method according to Claim 6 wherein the plant is a monocotyledonous species selected from wheat, rice, and sorghum.
8. A method according to Claim 6 further comprising  
35 the steps of:
  - a) culturing cells or tissues from the plant

b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a sequence coding for at least one peptide having the amino acid sequence of one of SEQUENCE I.D. NOS. 1 through 3 or 6 through 21, and

c) regenerating protected whole plants from the cell or tissue culture.

9. A method according to Claim 8 which comprises the further step of sexually or clonally reproducing the whole plant in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

10. A method according to Claim 8 in which the expression cassette is introduced into the cells by electroporation.

11. A method according to Claim 8 in which the expression cassette is introduced into the cells by microparticle bombardment.

12. A method according to Claim 8 in which the expression cassette is introduced into the cells by microinjection.

13. A method according to Claim 8 for providing resistance to microorganisms in *Agrobacterium tumefaciens*-susceptible plants in which the expression cassette is introduced into the cells by infecting the cells with *Agrobacterium tumefaciens*, a plasmid of which has been modified to include the expression cassette.

14. A method of imparting resistance to diseases caused by plant pathogens selected from *Fusarium graminearum*, *Fusarium moniliforme*, *Sclerotinia sclerotiorum*, *Sclerotinia trifoliorum*, and *Aspergillus flavus*, to plants of a taxon susceptible to those diseases, comprising the steps of:

a) selecting a fertile, disease resistant plant prepared by the method of Claim 10 from a sexually compatible taxon;



b) sexually crossing the disease resistant plant with a plant from the disease susceptible taxon;

c) recovering reproductive material from the progeny of the cross; and

5 d) growing disease resistant plants from the reproductive material.

15 15. A method according to Claim 14 for imparting disease resistance and antimicrobial activity in a taxon consisting of substantially homozygous plants, which comprises the further steps of repetitively:

a) backcrossing the disease resistant progeny with substantially homozygous, disease susceptible plants from the taxon; and

15 b) selecting for expression of disease resistance and antimicrobial activity along with the other desired characteristics of the susceptible taxon from among the progeny of the backcross, until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with disease resistance and antimicrobial  
20 activity.

16. An expression cassette comprising a DNA fragment which codes for a peptide having the amino acid sequence of SEQUENCE I.D. NOS. 1 through 3 or 6 through 21, operably linked to plant regulatory sequences which cause the  
25 expression of the DNA clone in plant cells.

17. An expression cassette comprising a DNA fragment which codes for a peptide having the amino acid sequence of SEQUENCE I.D. NOS. 1 through 3 or 6 through 21, operably linked to bacterial expression regulatory sequences which  
30 cause the expression of the DNA clone in bacterial cells.

18. Bacterial cells containing as a foreign plasmid at least one copy of an expression cassette according to Claim 17.

19. Transformed plant cells containing as foreign DNA  
35 at least one copy of the DNA sequence of an expression cassette according to Claim 164.

20. Transformed cells according to Claim 19, further characterized in being cells of a monocotyledonous species.

21. Transformed cells according to Claim 20, further characterized in being maize, sorghum, wheat or rice cells.

5 22. Transformed cells according to Claim 19, further characterized in being cells of a dicotyledonous species.

23. Transformed cells according to Claim 22, further characterized in being soybean, alfalfa, tobacco, rapeseed, sunflower or tomato cells.

10 24. A maize cell or tissue culture comprising cells according to claim 21.

25. A transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette according to Claim 16.

15 26. A antimicrobial composition for application to plants, comprising an antimicrobial amount of at least one peptide having the amino acid sequence of SEQUENCE I.D. NOS. 1 through 3 or 6 through 21, in a non-phytotoxic vehicle.

27. A composition according to Claim 26 wherein the  
20 vehicle is adapted for systemic administration to a plant.

## INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 94/14619

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/82 C07K14/435 C12N5/10 C12N1/21  
 A01H5/00 A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A01H A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages        | Relevant to claim No. |
|------------|---|-----------------------|
| A          | EP,A,0 545 730 (PIONEER HI-BRED) 9 June 1993<br>see the whole document<br>---             | 1-27                  |
| A          | EP,A,0 513 613 (SEIKAGAKU KOGYO CO LTD) 19 November 1992<br>see the whole document<br>--- | 1-27                  |
| A          | EP,A,0 497 366 (DONEGANI GUIDO IST) 5 August 1992<br>see page 15 - page 21<br>---         | 1-27                  |
| A          | WO,A,90 11771 (UNIV ROCKEFELLER) 18 October 1990<br>see the whole document<br>---         | 1-27                  |
|            | ---<br>-/--   |                       |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\* & \* document member of the same patent family

Date of the actual completion of the international search

19 April 1995

Date of mailing of the international search report

04.05.95

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 94/14619

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |   |                       |
|--|---|-----------------------|
| Category *   | Citation of document, with indication, where appropriate, of the relevant passages        | Relevant to claim No. |
| A  | EP,A,0 502 718 (PIONEER HI BRED INT) 9<br>September 1992<br>see the whole document<br>--- | 1-27                  |
| A  | WO,A,89 04371 (UNIV LOUISIANA STATE) 18<br>May 1989<br>see the whole document<br>-----    | 1-27                  |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/14619

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 3 and 4(both partially in so far as they relate to an in-vivo method) are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the
2. ☐ Claims Nos.: compound/composition.  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/14619

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) |          | Publication<br>date |
|---|---------------------|----------------------------|----------|---------------------|
| EP-A-0545730                              | 09-06-93            | AU-B-                      | 656442   | 02-02-95            |
|   |                     | AU-A-                      | 2978692  | 10-06-93            |
|   |                     | CA-A-                      | 2084346  | 07-06-93            |
|   |                     | JP-A-                      | 6113853  | 26-04-94            |
|   |                     | NZ-A-                      | 245217   | 27-06-94            |
| -----                                     |                     |                            |          |                     |
| EP-A-0513613                              | 19-11-92            | AT-T-                      | 113964   | 15-11-94            |
|   |                     | AU-A-                      | 1599892  | 05-11-92            |
|   |                     | DE-D-                      | 69200644 | 15-12-94            |
|   |                     | ES-T-                      | 2066510  | 01-03-95            |
|   |                     | WO-A-                      | 9219645  | 12-11-92            |
|   |                     | JP-A-                      | 5163298  | 29-06-93            |
|   |                     | OA-A-                      | 9696     | 30-08-93            |
| -----                                     |                     |                            |          |                     |
| EP-A-0497366                              | 05-08-92            | AU-B-                      | 648140   | 14-04-94            |
|   |                     | AU-A-                      | 1065092  | 06-08-92            |
|   |                     | CA-A-                      | 2060455  | 02-08-92            |
|   |                     | JP-A-                      | 5294995  | 09-11-93            |
| -----                                     |                     |                            |          |                     |
| WO-A-9011771                              | 18-10-90            | CA-A-                      | 2031199  | 13-10-90            |
|   |                     | EP-A-                      | 0422215  | 17-04-91            |
|   |                     | JP-T-                      | 3501742  | 18-04-91            |
| -----                                     |                     |                            |          |                     |
| EP-A-0502718                              | 09-09-92            | AU-A-                      | 1139992  | 10-09-92            |
|   |                     | JP-A-                      | 6228190  | 16-08-94            |
|   |                     | NZ-A-                      | 241814   | 25-11-94            |
| -----                                     |                     |                            |          |                     |
| WO-A-8904371                              | 18-05-89            | AU-A-                      | 2802989  | 01-06-89            |
|   |                     | CA-A-                      | 1321157  | 10-08-93            |
| -----                                     |                     |                            |          |                     |